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NOTE

Inhibitory effects of devil's claw (secondary root of *Harpagophytum procumbens*) extract and harpagoside on cytokine production in mouse macrophages

Kazunori Inaba · Kazuya Murata · Shunsuke Naruto · Hideaki Matsuda

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Abstract Successive oral administration (50 mg/kg) of a 50% ethanolic extract (HP-ext) of devil's claw, the secondary root of *Harpagophytum procumbens*, showed a significant anti-inflammatory effect in the rat adjuvant-induced chronic arthritis model. HP-ext dose-dependently suppressed the lipopolysaccharide (LPS)-induced production of inflammatory cytokines [interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α)] in mouse macrophage cells (RAW 264.7). Harpagoside, a major iridoid glycoside present in devil's claw, was found to be one of the active agents in HP-ext and inhibited the production of IL-1β, IL-6, and TNF-α by RAW 264.7.

Keywords Devil's claw · Harpagoside · Interleukin-1β · Interleukin-6 · Tumor necrosis factor

Introduction

The so-called devil's claw is a traditional South African medicine that has been used for pain relief and treatment of arthritis. Devil's claw is the secondary root of *Harpagophytum procumbens* DC. (Pedaliaceae), and harpagoside, an iridoid glycoside, is known to be a major constituent of devil's claw [1, 2]. There have been many reports regarding the anti-inflammatory effects of devil's claw extracts. Lanhers et al. [3] reported that the intraperitoneal (i.p.) administration of an aqueous extract (400 mg/kg) showed potent anti-inflammatory effects in rat carageenan-induced acute inflammation. Baghdikian et al. [4] reported that an aqueous extract possessed potent inhibitory activity only at high dose (400–800 mg/kg, i.p.) in a rat carageenan-induced acute inflammation model. Whitehouse et al. [5] reported that a Canadian herbal product of devil's claw (20–6,000 mg/kg) had no effect on rat carageenan-induced hind paw edema. In our preliminary experiments, a 50% ethanolic extract of devil's claw (HP-ext, 200 mg/kg, single oral administration, p.o.) showed no significant activity in the rat carageenan-induced acute inflammation model (data not shown). On the basis of these results, the anti-inflammatory effect of devil's claw on acute inflammation models has yet to be fully confirmed.

With regard to the activity of devil's claw extract in animal models of chronic inflammation, Whitehouse et al. [5] reported that the devil's claw (2 g/kg) described above showed no anti-inflammatory activity in a rat adjuvant-induced edema model. Anderson et al. [6] reported that a 60% ethanolic extract (100 mg/kg) showed complete inhibition in the rat Freund's adjuvant-induced edema model, although the administration route of test samples was ambiguous. These reports indicate that the efficacy of devil's claw extracts on chronic inflammation in rats is controversial.

Thus, as a part of our research program aimed at finding anti-inflammatory agents from natural sources, we examined whether oral administration of HP-ext has anti-inflammatory effects on adjuvant-induced arthritis in female SD rats. Adjuvant-induced arthritic rats have been recognized as a pathologic model of rheumatoid arthritis (RA) [7]. Inflammatory cytokines, such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α, are produced by lymphocytes and macrophages, and are involved in RA [8]. Since anti-cytokine therapy was recently recognized as an effective clinical treatment for RA, inhibition of cytokine production by macrophages may suppress an

K. Inaba · K. Murata · S. Naruto · H. Matsuda (✉)
Faculty of Pharmacy, Kinki University,
3-4-1 Kowakae, Higashi-osaka, Osaka 577-8502, Japan
e-mail: matsuda@phar.kindai.ac.jp

exacerbation of the inflammatory reaction in adjuvant-induced arthritic rats. However, there have been no reports dealing with the effects of devil's claw on the production of inflammatory cytokines by macrophages. In the present study, we examined the inhibitory effects of HP-ext on lipopolysaccharide (LPS)-induced production of the inflammatory cytokines IL-1β, IL-6, and TNF-α by mouse macrophage cells (RAW 264.7 cells).

Materials and methods

Plant material

Dry devil's claw (secondary root of *H. procumbens*) was provided by Nihon Prosperity Center Co., Ltd. (Osaka, Japan) in 2001. A voucher specimen (HP-0112) is deposited at Kinki University.

Preparation of HP-ext and isolation of harpagoside

Powdered devil's claw (300 g) was extracted with 50% ethanol (3 l, twice) for 2 h under reflux. The collected extract was concentrated under reduced pressure followed by lyophilization to give a 50% ethanolic extract (HP-ext, 184.2 g, 61.4% yield). Harpagoside (colorless amorphous powder, isolation yield, 1.23 g, 0.4% from the secondary root) was isolated from HP-ext according to the method described by Litchi et al. [9], and was identified by direct comparison of spectral data (mass spectra and ^{1}H- and ^{13}C-NMR) and optical rotation with those of an authentic sample (Extrasynthese, Genay Cedex, France).

Animals

Female SD rats (160–180 g) were purchased from CLEA Japan, Inc. (Tokyo, Japan). Rats were maintained in an air-conditioned room with lighting from 0700 to 1900 hours. Room temperature (about 23°C) and humidity (about 60%) were automatically regulated. Laboratory pellet chow (CLEA Rodent Diet CE-2, CLEA Japan, Inc., Tokyo, Japan) and water were freely available.

All experimental protocols were approved by the Committee for the Care and Use of Laboratory Animals at Kinki University, and conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Adjuvant-induced arthritis in rats

The arthritis model in rats was induced according to the method of Nakamura et al. [10]. Briefly, arthritis was induced by intradermal injection of a 0.05 ml suspension of dry heat-killed *M. butyricum* (Difco Laboratories, Detroit, MI, USA) (10 mg) in Bayol F (1 ml) as an adjuvant agent into the tail and right hind paw of SD rats (180–220 g). The right hind paw volume was measured periodically on days 0 (initial), 1, 3, 5, 7, 10, 14, 19, 24, and 30. Volume of edema was determined based on water displacement volume. Results are expressed as an increase in right hind paw volume, as compared with the initial right hind paw volume. Test substances and prednisolone, a positive control, were suspended in a 0.2% carboxymethylcellulose sodium (CMC-Na) solution (0.2 ml/100 g body weight of rat), and were administrated orally once daily for 30 days starting from the first day (day 0, adjuvant was injected on this day). A solution of 0.2% CMC-Na was administered (0.2 ml/100 g body weight of rats/day) to the control group and to the vehicle control rats.

Determination of IL-1β, IL-6, and TNF-α in mouse macrophage cells (RAW 264.7 cells)

A mouse macrophage-like cell line (RAW 264.7) was obtained from Dainippon Sumitomo Pharma Co., Ltd. (Osaka, Japan). Cells were cultured at 37°C in Dulbecco's modified Eagle medium (DMEM) (Gibco, Invitrogen Co., Staley Road, CA, USA) supplemented with 10% fetal bovine serum (Nichirei Biosciences Inc., Tokyo, Japan) and 1% antibiotic–antimycotic solution (mixture of 10,000 U/ml penicillin, 10,000 μg/ml streptomycin sulfate, and 25 μg/ml amphotericin B) (Gibco, Invitrogen Co.) in a humidified 5% CO_2 atmosphere.

For IL-1β determination, RAW 264.7 cells were seeded in a 24-well plate (COSTAR 3526, Corning International K.K., Corning, NY, USA) (6.0×10^5 cells/well); for IL-6 and TNF-α determination, cells were seeded in a 48-well plate (FALCON 353078, Becton–Dickinson Labware, Franklin Lakes, NJ, USA) (6.0×10^4 cells/well). After plates were pre-incubated for 2 h, cells were treated with test samples of various concentrations and were subsequently stimulated with lipopolysaccharide (LPS; *Escherichia coli* O111 B4) (1 μg/ml) (Sigma–Aldrich, St. Louis, MO, USA) for 24 h. Test samples and LPS were dissolved in 0.5% dimethyl sulfoxide (DMSO) in Dulbecco's phosphate buffered saline (Ca^{2+}- and Mg^{2+}-free, CMF-DPBS) (Gibco, Invitrogen Co.).

IL-1β, IL-6, and TNF-α levels in supernatants of RAW 264.7 cell culture medium were quantified by using ELISA kits (Pierce Biotechnology, Inc., Rockford, IL) according to the manufacturer's instructions. Data points represent mean values ± standard error ($n = 3$).

Assay for cell viability

RAW 264.7 cells (6.0×10^4 cells/well in 190 μl of DMEM) were seeded into 96-well plates (FALCON 353072, Becton–Dickinson Labware), and incubated at 37°C in a CO_2 incubator. After 2 h incubation, 5 μl of each sample solution and LPS (1 μg/ml) were added to each well in triplicate, and the plates were incubated for 20 h at 37°C in the CO_2 incubator. Test samples and LPS were dissolved in 0.5% DMSO in CMF-DPBS.

After incubation, cell viability was assayed by adding 5 μl of TetraColor ONE reagent solution (Seikagaku Co., Tokyo, Japan) to each well. After 4 h, optical density (OD) at 492 nm was measured for each well by using a microplate reader (Tecan Japan Co., Ltd., Kawasaki, Japan). Percentage of living cells in each well was calculated with respect to the OD value of living cells in the control group (100%).

Statistical analysis

Experimental data were tested for statistical significance by the Tukey–Kramer multiple range test (using StatView for Windows, Ver. 5.0; SAS Institute Inc., Cary, NC, USA, 1998).

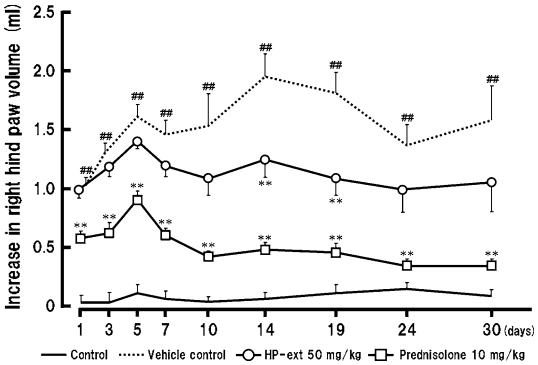

Fig. 1 Effects of HP-ext and prednisolone on right hind paw edema in adjuvant-induced arthritis in rats. Value represent mean ± SE of 10–11 rats. Significantly different from the control group, $^{\#\#}P < 0.01$; from the vehicle control group, $^{**}P < 0.01$

Results and discussion

The anti-inflammatory effects of successive oral administration of HP-ext and prednisolone on rat hind paw swelling in the adjuvant-induced chronic arthritis model are shown in Fig. 1. HP-ext (50 mg/kg, p.o.) showed no significant inhibitory activity on day 5 (early stage), but showed significant activity on days 14 and 19 (chronic stage). Prednisolone (10 mg/kg, p.o.), the positive control, showed potent inhibition of swelling at both the early and chronic stages. These results confirm that successive oral administration of HP-ext showed more potent anti-inflammatory effects at the chronic stage than at the acute stage in adjuvant-induced chronic arthritic rats. The inflammatory response in adjuvant-induced arthritic rats is a well-established model of RA, and RA is thought to be caused by inflammatory cytokines produced by macrophages. This prompted us to examine the inhibitory effects of HP-ext and harpagoside on LPS-induced production of the inflammatory cytokines IL-1β, IL-6, and TNF-α by RAW 264.7 cells.

As shown in Table 1, HP-ext showed potent inhibitory activity against IL-1β, IL-6, and TNF-α production at a concentration of 500 μg/ml. Harpagoside inhibited production of inflammatory cytokines without cytotoxicity at a concentration of 200 μM. Harpagoside was previously found to inhibit NO production [11]. However, to the best of our knowledge, this is the first report on the inhibitory effects of harpagoside on production of inflammatory cytokines.

In conclusion, it was confirmed that HP-ext exhibits anti-inflammatory effects at the chronic stage in the rat adjuvant-induced arthritis model. Part of the anti-inflammatory activity of HP-ext may be attributed to inhibition of the production of IL-1β, IL-6, and TNF-α by macrophages.

Table 1 Effects of HP-ext and harpagoside on LPS-induced production of IL-1β, IL-6, and TNF-α by RAW 264.7 cells

Sample	Conc. (μg/ml)	Inhibition (%)			Cell viability (%)
		IL-1β	IL-6	TNF-α	
Control	–	0.0 ± 6.4	0.0 ± 6.3	0.0 ± 6.6	100.0 ± 2.1
HP-ext	50	−23.1 ± 3.0*	9.3 ± 6.5	19.3 ± 12.5	111.1 ± 5.7
	200	27.4 ± 5.7**	49.2 ± 1.1**	47.9 ± 5.2**	108.0 ± 2.6
	500	87.4 ± 0.9**	83.0 ± 0.7**	74.4 ± 2.6**	96.9 ± 6.0
Harpagoside	50 (μM)	26.3 ± 7.3*	18.0 ± 0.5*	6.8 ± 3.0	101.3 ± 0.6
	100	51.2 ± 2.6**	19.7 ± 2.4*	37.9 ± 10.0*	102.6 ± 2.5
	200	42.3 ± 5.0**	45.3 ± 2.4**	56.1 ± 8.2**	102.8 ± 0.8

Each value represents the mean ± SE of 3 experiments. Significantly different from the control group, $^{*}P < 0.05$, $^{**}P < 0.01$

Harpagoside was found to be one of the active agents in HP-ext and inhibited production of IL-1β, IL-6, and TNF-α by macrophage RAW 264.7 cells.

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